CHOLINESTERASE AND LIPASE IN THE AMOEBOCYTES, INTESTINAL EPITHELIUM AND HEART MUSCLE OF THE QUAHOG, VENUS MERCENARIA [1]

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The isolated heart of the quahog, *Venus mercenaria,* has been shown by Prosser (1940) to be highly sensitive to acetylcholine and has been used by Welsh (1943) for the biological assay of acetylcholine. This preparation is particularly useful due to its great sensitivity to acetylcholine, resistance to changes in pH and rapid recovery. Thresholds for inhibition of the spontaneous beat of the isolated heart by acetylcholine are of the order of 10^{-10} to 10^{-11} gm./ml. Treatment of the heart with cholinesterase inhibitors (physostigmine, prostigmine or di-isopropyl fluorophosphate) results in a two to five times increase in inhibition of the strength of the heart beat when acetylcholine is added subsequently (Welsh and Taub, 1948). This slight potentiation is attributed to the low cholinesterase activity reported in the *Venus* heart by Jullien *et al.* (1938) and Smith and Glick (1939).

Augustinsson (1946a) showed that the tissues of the mollusc *Mya arenaria* hydrolyzed acetylcholine, benzoylcholine and acetyl β methyl choline and that the blood of the snail *Helix pomatia* hydrolyzes these substrates (1946b). Mendel, Mundell and Rudney (1943) reported that benzoylcholine is hydrolyzed by serum cholinesterase [3] but not by acetylcholinesterase or esterase and that acetyl-β-methyl choline is hydrolyzed only by acetylcholinesterase.

In a series of unpublished experiments concerned with the staining of the *Venus* heart with supravital dyes, questions arose regarding the localization of esterase activity in the heart, and the association of esterase activity with cell components which stained with Janus green B.

After staining whole *Venus* hearts in Janus green B 1 : 10,000 in sea water for two hours at 19° C., the ventricular and atrial muscle fibers were stained light blue-green. The cut end of the intestine was intensely stained, but little staining was observed in the circular muscle of the intestine. Scattered throughout the heart muscle, numerous amoebocytes were filled with dark blue-green granules. Reduction of the blue-green dye occurred when fragments of atrial muscle were spread under a cover slip on a microscope slide. The blue-green color of di-ethyl safranin dimethyl anilin (Janus green B) changed to the pink-red color of di-ethyl safranin,

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[3] Serum cholinesterase is synonymous with non-specific cholinesterase, pseudo-cholinesterase, type II cholinesterase, and s-type cholinesterase. Human serum, dog muscle, guinea pig liver, rat liver and numerous other tissues are capable of attacking benzoylcholine at various rates. Acetylcholinesterase is synonymous with specific cholinesterase, type I or true cholinesterase. Esterase is synonymous with non-specific esterase or aliesterase.

the reduction product. This indicates that hydrogen donor enzymes are probably present in the amoebocyte granules.

Structures which are known to contain esterase or cholinesterase and which stain with Janus green B occur in motor end-plates (Couteaux, 1947) and mitochondria (Zacks and Welsh, 1951). The appearance of intercostal muscle end-plates stained supravitally by Janus green B and for cholinesterase is similar. In both cases, the edges of the subneural apparatus of the end-plate are reactive. Rat liver mitochondria, which stain selectively with dilute solutions of Janus green B, also contain serum cholinesterase (Zacks and Welsh, 1951). The basis of this parallelism in staining may be due to the quaternary nitrogen groups of the basic dyes which mimic the "cationic head" of acetylcholine. Other evidence for the specific combination of Janus green B with cholinesterases and "receptor" substance of the *Venus* heart and frog end-plates occurs in the strong inhibitory actions of Janus green B on cholinesterase (Massart and Dufait, 1941) and the blocking activity of Janus green B and other basic dyes in the frog rectus abdominis preparation (unpublished observations).

The Janus green B staining granules of amoebocytes may well be regarded as mitochondria since they stain with this dye, contain hydrogen donor enzymes and stain with mitochondrial stains. Further investigations on the histochemistry of *Venus* amoebocytes will appear elsewhere.

The esterase and lipase activity of the amoebocytes and alimentary tract of several bivalve molluscs has been studied. Yonge (1926b) reported that amoebocytes from the oyster *Ostrea* were able to digest olive oil and fish erythrocytes, thus indicating the presence of intracellular lipase and protease. The ingestion and digestion of olive and peanut-oils were confirmed in the oyster, *Crassostrea virginica*, by George (1952). Takatsuki (1934) found difficulty in following the hydrolysis of olive oil in the amoebocytes of *Ostrea* but observed that methyl acetate was more readily hydrolysed. This finding probably indicates the presence of esterase. Yonge (1923) reported that lipolytic enzymes are absent in the midgut of the clam *Mya*. Details of the physiology and biochemistry of amoebocytes may be found in the work of Yonge (1923, 1926a, 1926b, 1929, 1946), Ohuye (1938), and the review of Haughton (1934).

In view of the early reports of lipase and esterase in amoebocytes and intestine of various molluscs and the selective staining of amoebocyte mitochondria by Janus green B, an investigation of the enzymes present in the isolated *Venus* heart, amoebocytes and intestine was of interest.

This investigation is divided into two parts. The first is concerned with evidence of enzymatic activity in the *Venus* heart and isolated amoebocytes obtained by manometric and biological methods. The second part presents evidence of enzymatic activity obtained through the use of recently developed histochemical methods.

Part I

Methods

Five *Venus* hearts were excised with an enclosed length of intestine and blotted to remove surface moisture. The combined net weight was 728 mg. The hearts were then homogenized for 25 seconds in 30 ml. of sea water in a Waring Blendor.

A modified version of the standard (Ammon, 1934) manometric procedure for the determination of cholinesterase activity was used. Warburg vessels with side arms were filled with 3 ml. of a solution composed of 25 ml. sea water, 5 ml. 1.26% $NaHCO_3$ and 0.5 ml. of a solution of acetylcholine bromide 8 mg./ml. or benzoylcholine 8 mg./ml. The final acetylcholine concentration in each vessel was 1 mg./ml. Aliquots (0.5 ml.) of the whole heart homogenate were added to the side arm of the flasks. Another 0.5-ml. aliquot of homogenate was dried at 60° C. for 24 hours and the true dry weight was obtained by subtracting the weight of the salts in the aliquot. Four vessels were filled by the method described above and automatic correction of spontaneous hydrolysis was provided by employing a thermobarometer containing substrate but not homogenate. The vessels were equilibrated 10 minutes at 21° C., gassed with a mixture of 95% nitrogen and 5% carbon dioxide for three minutes and re-equilibrated for another 10 minutes. At the end of this period, the homogenate was tipped in and readings taken at 10-minute intervals for 60 minutes. Q_{Ach} values were calculated on the basis: microliters CO_2/mg. dry weight × hr.

Results

Since the cholinesterase activity of the hearts is variable and the relatively low activity is on the threshold of accurate detection by this procedure, a sample experiment will be reported and the average results considered (Table I).

Table I

		Acetylcholine bromide 1 mg./ml.	Benzoylcholine 1 mg./ml.
Vessel	I	2.2	4.6
	II	9.0	7.9
	III	3.2	4.0
	IV	5.1	—
		Average: 4.8 (Q_{Ach} 0.61)	5.5 (Q_{Bzch} 0.78)

As can be seen from Table I, these average values ($Q_{Ach} = 0.61$; $Q_{Bzch} = 0.78$) are quite low compared with a Q_{Ach} of 34 for horse serum (Mendel and Rudney, 1943) or the average Q_{Ach} of 1.42 for isolated rat liver mitochondria (Zacks and Welsh, 1951). Also, the average values found in this study are lower than the Q_{Ach} of 5.5 reported by Smith and Glick (1939) calculated on the basis of microliters of CO_2/30 minute × mg. weight. According to the criteria of Mendel, Mundell and Rudney (1943), the splitting of benzoylcholine by *Venus* heart homogenates indicates the presence of serum cholinesterase. Acetylcholinesterase and esterase do not attack benzoylcholine according to these authors.

The confirmation of low serum cholinesterase activity in *Venus* heart homogenates was achieved, but the localization of the enzyme in the several tisuses included in the homogenate required further investigation. In practice, a length of intestine as well as numerous amoebocytes is included in the heart preparation used for the assay of acetylcholine and in the whole heart homogenates, so that attention must be directed to these component tissues.

In order to determine if the numerous amoebocytes in the heart contributed to the cholinesterase activity of the homogenates, isolated and washed amoebocytes were prepared and assayed for cholinesterase activity.

Five ml. of *Venus* blood were obtained by ventricular aspiration of several quahogs. The pooled blood was centrifuged at 700 × g. for 10 minutes to sediment the amoebocytes. The supernatant was decanted and stored at 4° C. in the refrigerator until it could be assayed. The sedimented amoebocytes were taken up in sea water and re-sedimented in order to wash away adhering serum. This procedure was repeated and the sediment was diluted with 5 ml. sea water. To 7 ml. of sea water, two ml. amoebocyte concentrate and one ml. 10^{-4} *M* acetylcholine bromide were added. This mixture was incubated at 27° C. for two hours and 0.1-ml. aliquots were tested on the *Venus* heart preparation (Welsh and Taub, 1948). Similarly, aliquots of mixtures containing separated serum were also tested. Control incubation mixtures were prepared which contained 6 ml. sea water, two ml. amoebocyte concentrate, one ml. physostigmine salicylate 10^{-3} *M* and one ml. acetylcholine bromide 10^{-4} *M*. After two hours incubation, mixtures were added to regularly

TABLE II

Summary of the composition of incubation mixtures used to detect cholinesterase activity

Number	(Ach) in mixture	Aliquot ml.	(Ach) in heart bath	Serum in mixture	Amoebocytes in mixture ml.	Physostigmine *M*	% inhibition
1	—	—	10^{-8} *M*	—	—	—	100
2	10^{-5} *M*	0.1	10^{-7}*	—	2	—	0
3	—	—	10^{-7}	—	—	—	100
4	10^{-5}	0.1	10^{-7}*	—	2	—	0
5	10^{-5}	0.1	10^{-7}*	—	2	10^{-4}	100
6	10^{-5}	0.1	10^{-7}*	—	2	—	0
7	10^{-5}	0.1	10^{-7}*	—	2	—	0
8	10^{-5}	0.1	10^{-7}*	2	—	—	0
9	10^{-5}	1.0	10^{-6}*	2	—	—	14
10	—	—	10^{-8}	—	—	—	100

* If there had been no hydrolysis of acetylcholine.

beating *Venus* hearts in a bath containing 9 ml. sea water. The final concentration of acetylcholine was 10^{-5} *M* in the control mixtures. The concentration of acetylcholine added to the incubation mixture was chosen so that the final concentration in the heart bath, 10^{-7} *M*, if there had been no hydrolysis of acetylcholine, would produce complete inhibition of the heart beat. In a typical experiment, test concentrations of acetylcholine were added to the bath containing the beating heart to measure the sensitivity of the preparation. After washing and a 5-minute rest period, test aliquots of the incubation mixtures were added to the baths. After washing and rest, control concentrations of acetylcholine were added to the baths to test the recovery of the test hearts.

Table II summarizes the manner in which the various incubation mixtures in a sample experiment were prepared. The effect of the various mixtures on the test hearts can be seen by comparing the number of the mixture in Table II with the corresponding number in Figure 1.

Figure 1 is a typical record of a sample experiment. It can be seen that the amoebocyte concentrate and the supernatant serum produced nearly complete hy-

drolysis of the acetylocholine present in the incubation mixtures in two hours as evidenced by the failure of test aliquots to inhibit the spontaneous beat of the isolated heart. The same final concentration of acetylcholine (10^{-7} *M*) added to the bath and aliquots of incubation mixtures protected with 10^{-4} *M* physostigmine salicylate, completely inhibited the beat of the *Venus* hearts tested. Test concentrations of acetylcholine (10^{-7} *M*), added to the test hearts after the experimental aliquots had been tested, showed that the test hearts were still sensitive to acetylcholine.

Thus, isolated and washed *Venus* amoebocytes as well as serum contain enzymes capable of hydrolyzing acetylcholine. The observed complete inhibition of these enzymes by physostigmine salicylate 10^{-4} *M* indicates the presence of cholinesterase, since lipase and esterase are slightly inhibited by this concentration of physostigmine (Easson and Stedman, 1937; Richter and Croft, 1942). The presence of lipase or esterase in *Venus* amoebocytes cannot be excluded without additional evi-

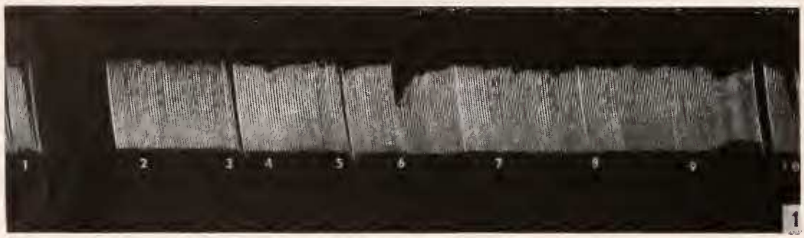


FIGURE 1. Sample record of the response of the isolated Venus heart to aliquots of incubation mixtures, to detect the presence of acetylcholine.

dence. The histochemical data, which will be presented below, indicate that a mixture of enzymes exists in the amoebocytes as well as in the intestine and heart muscle.

PART II

In order to obtain more information concerning the intracellular localization of esterases, recently developed histochemical methods were applied to amoebocytes, intestinal epithelium and heart muscle of *Venus*. These procedures have been used in combination with various enzyme inhibitors in an attempt to characterize the types of enzymes present.

METHODS

Nachlas and Seligman (1949) developed a procedure for the histochemical localization of esterase and lipase in acetone-fixed tissues. This method is based on the hydrolysis of β-naphthyl acetate by esterase and lipase. When β-naphthyl acetate is hydrolyzed, the resulting product is spontaneously converted to β-naphthol which, in the presence of a stabilized diazotised salt, precipitates as a granular, insoluble pigment at or near the site of enzymatic activity. Later, it was found that β-naphthyl acetate also was attacked by the enzymes of fresh, unfixed nervous tissue and that the failure of nervous tissue to react in the original method was due to the destruction of acetylcholinesterase by acetone. The modified procedure (Ravin, Zacks and

Seligman, 1953) utilizes the 6-brom derivative of β-naphthyl acetate as the substrate and tetrazotized anisidine (diazo blue B) as the coupling agent in the staining of fresh frozen sections cut by the Linderstrøm-Lang method as modified by Coons *et al.* (1951). Sections cut at 10 micra can be stored at 4° C. for one or two weeks without marked decline in enzymatic activity. When applied to fresh, frozen sections, this method will demonstrate lipase, esterase and cholinesterases. The particular enzyme present is determined by pre-treatment of the sections for one hour in various inhibitors known to differentiate lipase, esterase and cholinesterases.

Sodium taurocholate (10^{-2} *M*, 10^{-3} *M*) was used to differentiate lipase from esterase since, according to Willstatter and Memmen (1924) and Nachlas and Seligman (1949), esterase is inhibited, but lipase is activated or unaffected by this reagent. Advantage was taken of the observations that acetylcholinesterase and serum cholinesterase are inhibited by low concentrations of physostigmine (10^{-5} *M*) while esterase is little affected by concentrations as great as 10^{-3} *M* (Easson and Stedman, 1937; Richter and Croft, 1942). Also cold acetone (4° C.) applied for 24 hours destroys cholinesterase activity but lipase and esterase activity are relatively unaffected (Nachlas and Seligman, 1949). Other inhibitors which are claimed to differentiate between the various enzymes considered were also used.

Quinine (5×10^{-2} *M*) was used by Nachlas and Seligman (1949) who found that pancreatic hydrolysis of β-naphthyl acetate was moderately inhibited as was esterase activity in liver and kidney. These authors conclude that quinine, in this concentration, inhibits lipase more than esterase. Also, Augustinsson (1948) reported that quinine hydrochloride (3.78×10^{-4} *M*) strongly inhibited the hydrolysis of choline esters and tributyrin catalyzed by serum and liver preparations. Due to this lack of clearly specific inhibition produced by quinine, this reagent is less valuable for the characterization of the enzymes present in the *Venus* heart. Quinine alkaloid was used in a concentration of 10^{-3} *M*.

Arsenilic acid (atoxyl) 10^{-1} *M* was used by Nachlas and Seligman (1949) to inhibit esterase in homogenates of rat kidney, liver and pancreas and other tissues. The high concentration required attests to the low specificity of this reagent. Thus, to differentiate the main recognized groups of enzymes (lipase, esterase and cholinesterase), reliance was placed on the use of sodium taurocholate, physostigmine and cold acetone as the most reliable criteria available.

As a check on the accuracy of localization of enzymatic activity demonstrated by the use of 6-brom-2-naphthyl acetate, the method of Barrnett and Seligman (1951) was also used. In this procedure, the substrate employed is indoxyl acetate, which, when hydrolyzed by lipase, esterase or cholinesterase, yields indoxyl which oxidizes in air to form an insoluble precipitate of indigo at or near the site of enzymatic activity. The enzymes which attack this substrate are the same as those which hydrolyze 6-brom-2-naphthyl acetate and therefore must also be controlled by the use of inhibitors.

To test for the presence of serum cholinesterase, the method of Ravin, Tsou and Seligman (1951) was used. This procedure employs carbonaphthoxycholine iodide as the artificial chromogenic substrate. The coupling agent (diazo blue B) is the same as that used with 6-brom-2-naphthyl acetate. Carbonaphthoxycholine is hydrolysed by serum cholinesterase, but not by esterase, lipase or acetylcholinesterase.

The histochemical methods described above were applied to fresh whole hearts from which thin spreads were made of atrial muscle or to frozen sections, cut at 10 micra in the Linderstrøm-Lang cryostat. The sections were incubated in a Michaelis barbital buffer (pH 7.4 for 6-brom-2-naphthyl acetate and carbonaphthoxycholine and pH 8.4 for indoxyl acetate) containing the substrate, diazo blue B, NaCl and $CaCl_2$ as recommended in the respective methods. In inhibition experiments, the inhibitors were made up in the proper buffer mixture and slides were incubated in the solutions for 60 minutes. At the end of this time, the inhibitor solution was poured off into a flask and substrate and coupling agent were added. The mixture was then filtered back into the coplin jar containing the sections. In all cases, the reaction was carried out in the presence of the inhibitor to avoid reversibility of inhibition due to washing out of the inhibitor. The reaction was allowed to proceed for 20 minutes and the slides were washed in tap water and mounted in Kaiser's glycerogel. The slides were examined as soon as possible after staining since deterioration of the preparations occurred.

Results and Discussion

Table III summarizes the results obtained in the histochemical examination of *Venus* amoebocytes, intestinal epithelium and heart muscle.

Table III

Hydrolysis of histochemical substrates by Venus amoebocytes, intestinal epithelium and heart muscle

Substrate	Amoebocytes	Intestinal epithelium	Heart muscle
6-brom β-naphthyl acetate	++	++	++
Indoxyl acetate	++	++	++
Carbonaphthoxycholine	++	+	++

After reaction with 6-brom-2-naphthyl acetate or indoxyl acetate, the amoebocytes were filled with blue or blue-violet punctate granules. Red-violet granules were seen in the amoebocytes after reaction with carbonaphthoxycholine iodide. The intestinal epithelium, especially the middle and basal regions of the tall columnar cells, was strongly reactive in many of the intestines examined as was the ventricular and atrial musculature. In other intestines, relatively little enzymatic activity was observed even though amoebocytes and heart muscle reacted normally. Apparently, these differences of enzyme activity in the intestinal epithelium reflect functional phases probably associated with digestion. Nuclei of amoebocytes, intestinal epithelium cells and heart muscle were never reactive.

Table IV summarizes the results obtained in the inhibition experiments. After pre-treatment with sodium taurocholate (10^{-3} *M*), no inhibition of amoebocytes or intestinal epithelium was observed although enzymatic activity in the ventricular muscle was partially inhibited. When a higher concentration of sodium taurocholate (10^{-2} *M*) was used, the intestinal epithelium and amoebocytes were still reactive, but the ventricular muscle enzymatic activity was completely inhibited. In a few experiments, it appeared that the reaction in the amoebocytes and intestinal epithelium

after sodium taurocholate (10^{-3} *M*) had been increased and that activation of the enzymes present had occurred.

Physostigmine alkaloid (10^{-3} *M*) produced complete inhibition of all reactive tissues, but the same inhibitor tested in lower concentration (2×10^{-5} *M*) failed to inhibit the amoebocytes but did somewhat reduce the intensity of the reaction in the intestinal epithelium and more intensely inhibited enzymatic activity in the ventricular muscle. The results obtained with physostigmine (10^{-3} *M*) should be regarded with some doubt since the addition of diazo blue B to a physostigmine solution of this concentration results in the production of a deep orange solution which suggests that a reaction has taken place which may prevent diazo blue B from subsequently coupling with the substrate.

Acetone (4° C.) applied for 24 hours slightly inhibited the reaction in the intestinal epithelium and amoebocytes but markedly decreased the enzymatic activity of the ventricular muscle.

TABLE IV

Inhibition of Venus amoebocytes, intestinal epithelium and heart muscle by esterase and lipase and cholinesterase inhibitors

Inhibitor Substrate 6-brom β-NA	Amoebocytes	Intestinal epithelium	Heart muscle
Sodium taurocholate 10^{-3} *M*	++	+++	+
Sodium taurocholate 10^{-2} *M*	+	++	−
Quinine alkaloid 10^{-3} *M*	++	++	+
Physostigmine alkaloid 2×10^{-5} *M*	++	+	±
Physostigmine alkaloid 10^{-3} *M*	−	−	−
Atoxyl 10^{-1} *M*	−	−	−
Acetone 24 hr., 4° C.	+	++	±
Water 90° C., one min.	−	−	−

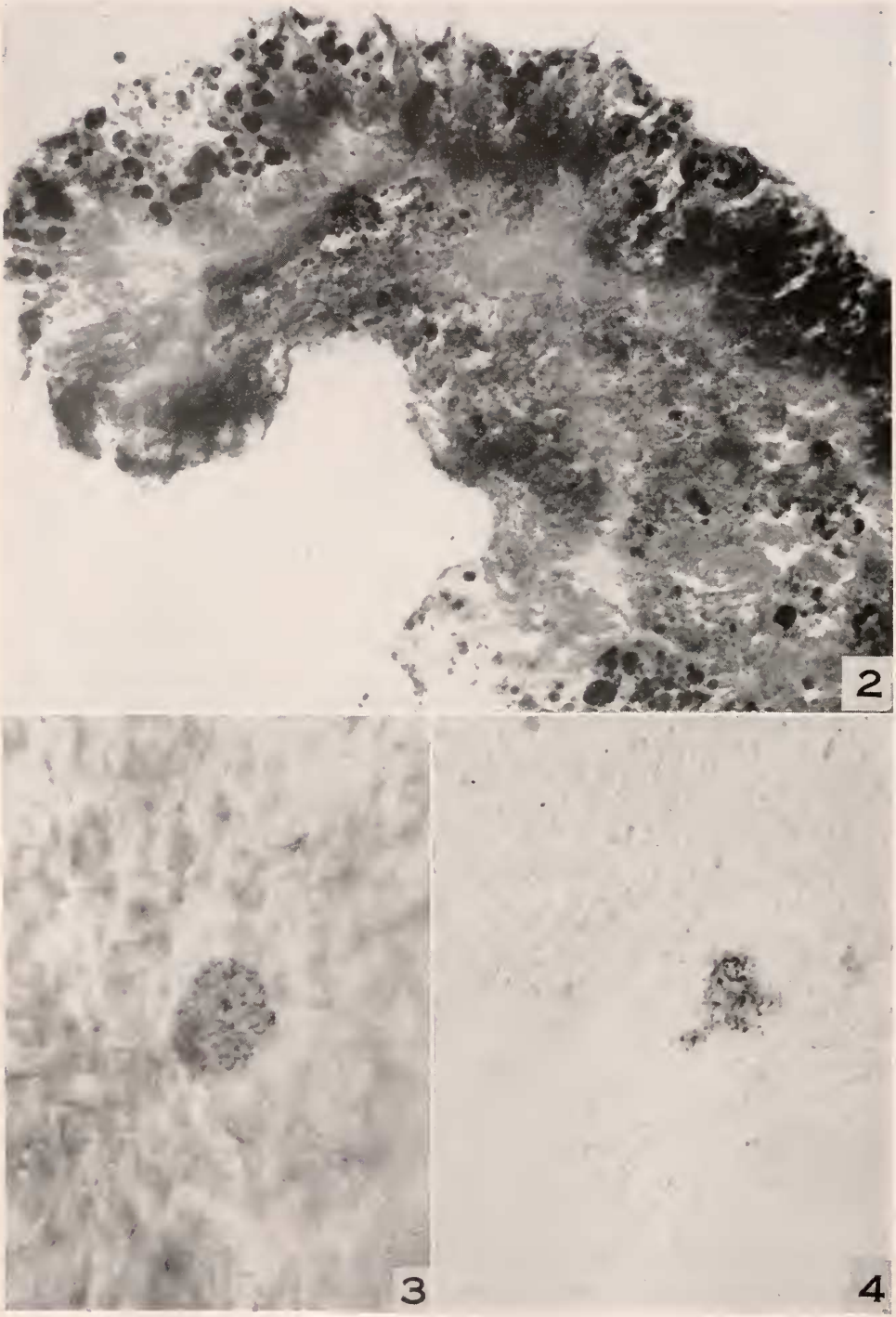
− Complete inhibition.
± Mostly inhibited, but few blue-violet granules present; considerably less than control.
+ Pink background, scattered blue-violet granules; less than control.
++ Fairly intense blue or blue-violet, punctate granulation; control level of staining.
+++ Very intense reaction; greater than control—activation.

Quinine alkaloid (10^{-3} *M*) failed to inhibit the amoebocytes or intestinal epithelium, but slightly decreased the reactivity of the ventricular muscle.

Arsenilate (10^{-1} *M*) completely inhibited all staining in the amoebocytes, intestinal epithelium and ventricular muscle.

When carbonaphthoxycholine iodide was used as the substrate, amoebocytes, intestinal epithelium and ventricular muscle were stained. The ventricular muscle was particularly reactive as indicated by the intense stain.

From the collected histochemical evidence summarized in Tables III and IV, it appears that lipase and serum cholinesterase are present in varying amounts in *Venus* amoebocytes, ventricular muscle and intestinal epithelium. The staining with carbonaphthoxycholine and the inhibition of staining with 6-brom-2-naphthyl acetate after pre-treatment with physostigmine (2×10^{-5} *M*), quinine (10^{-3} *M*) and cold acetone indicate that the activity of serum cholinesterase is greater in the ventricular muscle than in the amoebocytes or intestinal epithelium. Lipase activity is



FIGURES 2–4.

localized in the intestinal epithelium and in the amoebocytes and to a lesser extent in the ventricular muscle. This is shown by the failure of sodium taurocholate to inhibit amoebocyte and intestinal epithelium activity. Ventricular muscle was completely inhibited by 10^{-2} *M* sodium taurocholate. Lipase activity in the intestinal epithelium was not destroyed by cold acetone, while the ventricular muscle was completely inhibited.

The histochemical findings summarized above demonstrate the distribution of serum cholinesterase and lipase activity in the *Venus* heart and associated tissues, and account for the results recorded in the literature and in this study. The demonstration of lipase in amoebocytes by histochemical means confirms the observations of Yonge (1926b) who observed the digestion of oil and fish erythrocytes in *Ostrea*. The hydrolysis of acetylcholine and benzoylcholine by whole heart homogenates can be attributed to serum cholinesterase as demonstrated by carbonaphthoxycholine iodide. It has been shown by Ravin *et al.* (1951) that β naphthyl acetate is hydrolyzed by homogenates of rat brain, rat liver, washed human erythrocytes and by human serum and purified human serum cholinesterase but that carbonaphthoxycholine iodide is hydrolyzed only by human serum and purified serum cholinesterase. Ravin *et al.* (1951) point out the similarity of carbonaphthoxycholine and benzoylcholine as the basis for the hydrolysis of both substrates by serum cholinesterase. It is interesting that rat liver fails to attack carbonaphthoxycholine yet contains an enzyme capable of hydrolyzing benzoylcholine which is associated with the mitochondria (Zacks and Welsh, 1951). This may indicate that more than one type of serum cholinesterase exists and that the type found in *Venus* is more like the type found in human serum than the enzyme present in rat liver mitochondria. Takatsuki's observation (1934) that amoebocytes of *Ostrea* contain enzymes capable of hydrolysing methyl acetate may be interpreted in terms of serum cholinesterase activity or an esterase. The presence of esterase in the heart and intestinal epithelium of *Venus* can not be completely excluded on the basis of the histochemical experiments. However, the nearly complete protection of acetylcholine by physostigmine (10^{-4} *M*) in incubation mixtures containing isolated amoebocytes suggests that serum cholinesterase is the enzyme in the amoebocytes which attacks acetylcholine most readily.

The role of amoebocytes in intracellular digestion has been studied by Yonge (1923, 1926a, 1926b, 1946) who believes that the digestion of fat and proteins in molluscs is primarily intracellular in the cells of the digestive diverticula and in the

FIGURES 2–4.

FIGURE 2. Fresh frozen transverse section through the intestine of *Venus mercenaria*, showing total enzymatic activity after 20 minutes incubation in a solution containing 10 mg. of 6-brom-2-naphthyl acetate and 40 mg. of diazo blue B in barbital buffer pH 7.4. Note the intense activity in the basal and midportions of intestinal epithelium. The dark globules of "excretory substance" do not contain enzymatic activity but are colored red by dissolved azo dye. Enzymatic activity in the epithelium is indicated by fine blue punctate granules of the precipitated dye. 100 ×.

FIGURE 3. View of a rounded-up amoebocyte, from a fresh spread of atrial muscle, containing granules of precipitated azo dye after 20 minutes staining with a mixture of β-naphthyl acetate and diazo blue B. 1000 ×.

FIGURE 4. An example of an amoebocyte with a characteristic pseudopod in a fresh spread of atrial muscle stained supravitally by β-naphthyl acetate and diazo blue B for 20 minutes at room temperature. 1000 ×.

amoebocytes. Thus, the amoebocytes are thought to have a major role in the absorption and digestion of fat and protein. Evidence of extracellular digestion of fats and proteins in the intestinal tract of several molluscs has been obtained by Sawano (1929), Mansour (1946) and Mansour-Bek (1946). George (1952), using Nile blue sulphate as an indicator, observed that oil droplets are hydrolyzed in the stomach of *Crassostrea virginica* and that neutral fat was hydrolyzed by minced crystalline style and digestive gland. This author was also able to confirm the ingestion and subsequent intracellular digestion of oil by the amoebocytes but concluded that the fat ingested was too little to be significant in nutrition.

The demonstration of variable levels of lipase and cholinesterase activity in the intestinal epithelium of *Venus* seems to lend support to the concept of extracellular digestion of fats in the intestinal tract of certain molluscs.

The role of serum cholinesterase, either in sera or tissues, is not known, although a digestive role might be suspected in *Venus* since the enzyme occurs in amoebocytes and intestinal epithelium.

Summary

1. Whole *Venus* heart homogenates hydrolyze acetylcholine and benzoylcholine at a low rate.

2. The enzymatic activity of such homogenates is the sum of that contributed by amoebocytes, intestinal epithelium and heart muscle.

3. Isolated amoebocytes hydrolyze acetylcholine and the enzyme responsible is inhibited by 10^{-4} *M* physostigmine, thus indicating the presence of cholinesterase.

4. Recently developed histochemical procedures demonstrate serum cholinesterase and lipase in amoebocytes, intestinal epithelium and heart muscle.

5. Greatest lipase activity is present in the amoebocytes and intestinal epithelium, while serum cholinesterase activity is greatest in the ventricular muscle.

6. The potentiation of the action of acetylcholine on the isolated *Venus* heart by physostigmine is due to inhibition of serum cholinesterase in the amoebocytes and especially in the heart muscle.

7. The presence of varying levels of lipase in the intestinal epithelium of *Venus* lends support to the suggested presence of extracellular lipolytic enzymes in certain molluscs.

8. In *Venus* amoebocytes, another case is seen where cholinesterase activity is associated with structures which stain supravitally with Janus green B.

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